

# Association of Blood Arsenic Levels with Increased Reactive Oxidants and Decreased Antioxidant Capacity in a Human Population of Northeastern Taiwan

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Arsenic is a notorious environmental toxicant known as both a carcinogen and an atherogen in human beings, but the pathogenic mechanisms are not completely understood. In cell culture studies, trivalent arsenic enhanced oxidative stress in a variety of mammalian cells, and this association may be closely associated with the development of arsenic-related diseases. To investigate the effect of arsenic exposure on oxidative stress in humans, we conducted a population study to determine the relationships of blood arsenic to reactive oxidants and antioxidant capacity at the individual level. We recruited 64 study subjects ages 42–75 years from residents of the Lanyang Basin on the northeast coast of Taiwan, where arsenic content in well water varies from 0 to  $\geq 3,000$   $\mu\text{g/L}$ . We used a chemiluminescence method, with lucigenin as an amplifier for measuring superoxide, to measure the plasma level of reactive oxidants. We used the azino-diethyl-benzthiazoline sulphate method to determine the antioxidant capacity level in plasma of each study subject. We determined arsenic concentration in whole blood by hydride formation with an atomic absorption spectrophotometer. The average arsenic concentration in whole blood of study subjects was  $9.60 \pm 9.96$   $\mu\text{g/L}$  ( $\pm$  SD) with a range from 0 to 46.50  $\mu\text{g/L}$ . The level of arsenic concentration in whole blood of study subjects showed a positive association with the level of reactive oxidants in plasma ( $r = +0.41$ ,  $p = 0.001$ ) and an inverse relationship with the level of plasma antioxidant capacity ( $r = -0.30$ ,  $p = 0.014$ ). However, we found no significant association ( $p = 0.266$ ) between levels of plasma reactive oxidants and antioxidant capacity. Our results also show that the lower the primary arsenic methylation capability, the lower the level of plasma antioxidant capacity ( $p = 0.029$ ). These results suggest that ingestion of arsenic-contaminated well water may cause deleterious effects by increasing the level of reactive oxidants and decreasing the level of antioxidant capacity in plasma of individuals. Persistent oxidative stress in peripheral blood may be a mechanism underlying the carcinogenesis and atherosclerosis induced by long-term arsenic exposure. **Key words:** antioxidant capacity, azino-diethyl-benzthiazoline sulfate method, blood arsenic, chemiluminescence, population study, reactive oxidants, superoxide. *Environ Health Perspect* 109:1011–1017 (2001). [Online 26 September 2001]

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Arsenic, a ubiquitous element present in the environment, is the main constituent of more than 200 mineral species on earth (1). In addition to its natural occurrence in mineral deposits, arsenical compounds are used in many human activities such as manufacturing, agriculture, and medicine (2). Arsenical compounds are transported into the environment mainly by water from wells drilled into arsenic-rich geologic strata or by ambient air during the smelting and burning of coal (1,2). However, the main route of arsenic exposure for the general population is via drinking water (2,3). Epidemiologic studies have documented that long-term exposure to inorganic arsenic (InAs) is associated with an increased risk of cancer of the lung, skin, and probably other anatomic sites (4,5). InAs is also one of major risk factors for blackfoot disease (BFD), a unique peripheral vascular disease identified in endemic areas of arseniasis in Taiwan where residents had used high-arsenic-tainted artesian well

water for more than 50 years (6). In addition, cardiovascular disease, such as ischemic heart disease and coronary heart disease (7,8), and cerebrovascular accidents (9) are also closely related to long-term ingestion of high-arsenic drinking water. Arsenic is an unusual environmental toxicant in that it induces carcinogenesis as well as atherosclerosis in human beings. This dual effect of arsenic may itself suggest a common mechanism shared by the pathogenic process of both diseases in their relation to arsenic exposure. A common pathogenic process involving cell proliferation has been suggested for the human carcinogenicity and atherogenicity of long-term exposure to InAs (10). However, the detailed mechanisms by which arsenic causes both cancer and atherosclerotic lesions in humans are not clear.

In *in vitro* studies, arsenite, a trivalent InAs compound, has been shown to induce morphologic transformation (11), structural and numeric chromosome changes (12,13),

sister chromatid exchanges (14), gene amplification (15), and micronuclei formation (16,17). Arsenite by itself does not induce gene mutations, but it was shown to potentiate the cytotoxicity, mutagenicity, and clastogenicity of several DNA-damaging compounds (18–20). Barchowsky et al. (21) recently showed that at noncytotoxic concentrations, arsenite increases DNA synthesis in cultured porcine vascular endothelial cells, an indication of a mitogenic response. The detailed mechanisms of arsenic genotoxicity and mitogenicity in cultured cells require further elucidation. Recent studies have indicated that arsenite may interfere with signal transduction pathways either by direct kinase/phosphatase-enzyme inhibition or by redox control of the regulatory molecules (21–24). Furthermore, generation of reactive oxidants during arsenic metabolism can play an important role in arsenic-induced injury (16,25–28). These studies suggest involvement of oxidative stress in the pathogenic effects of arsenic exposure.

Oxidative stress, which results when oxygen free radical generation exceeds the body's antioxidant defense, has been conventionally considered to have implications in the pathophysiology of several human diseases, including cancer and atherosclerosis (29–31). To investigate the effect of arsenic exposure on oxidative stress at the individual level, we examined reactive oxidants and antioxidant capacity in plasma of an arsenic-exposed yet apparently healthy population, and determined the relationships of the levels of reactive oxidants and antioxidant capacity in

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plasma to the arsenic concentration in whole blood of subjects. We also examined influences of lifestyle characteristics and arsenic metabolism capability on risk associations for any possible confounding effect.

## Methods

**Study area.** In Taiwan, well water with high arsenic levels is clustered in the Lanyang Basin and in the so-called BFD-endemic area in southwestern Taiwan (32). The Lanyang Basin of Ilan County is located on the north-eastern coast of Taiwan. The arsenic concentration in well water from the Lanyang Basin area ranges from undetectable to over 3,000 µg/L, and over 50% of surveyed wells contained a level of arsenic below 50 µg/L (33). Although most of the residents in the Lanyang Basin use household-owned well water as their primary drinking source, arsenic-associated cancers observed in the BFD-endemic area of southwestern Taiwan have not yet been found extensively in the Lanyang Basin. However, a high prevalence of cerebrovascular diseases associated with long-term arsenic exposure has recently been reported in the Lanyang Basin (9).

**Subject recruitment and blood collection.** We focused on two villages—Meicheng and Meifu in Chuangwei Township, Ilan County—where residents currently use household-owned water supply wells as their main drinking source. The total population age 40 years or over in the two villages was approximately 1,000 in 1995. Since then, the population has been regularly followed up for health status (33). The entire population has spent most of their lives in their respective villages (33). The subjects for the present study were recruited from this population.

We first classified the population into four groups according to the arsenic level in their household well water:  $\leq 10$  µg/L, 10.1–50 µg/L, 50.1–300 µg/L, and  $> 300$  µg/L. Eighty study subjects were grouped by exposure level, each group containing 20 individuals about the same age and equal in sex distribution to those in the other groups. Each subject was scheduled for a health examination in a local hospital. During the hospital visit, each was first asked for consent to join this study. Only study subjects who gave their consent were recruited for blood collection and were given a questionnaire-interview by a nurse in the hospital. All subjects recruited for this study were free of any clinical symptoms such as inflammatory diseases. For each study subject, a 10-mL blood sample was collected into a heparinized and aluminum foil-wrapped blood tube under fasting condition. We stored 3 mL blood sample at  $-20^{\circ}\text{C}$  for use to detect arsenic content in whole blood as a measurement of the most recent exposure to arsenic exposure.

The analysis was generally performed within 2 weeks. We centrifuged 2 mL sample to separate plasma from packed cells for subsequent assays of reactive oxidative species and total antioxidant capacity within 6 hr after collection. We interviewed subjects using a formatted questionnaire to obtain relevant information, including demographic and lifestyle characteristics such as cigarette smoking and alcohol and tea consumption, as well as daily sources of drinking water. Only current users of household-owned well water were included in this study. All study subjects were enrolled between November 1997 and May 1998.

**Determination of arsenic concentration in whole blood.** We determined arsenic content in whole blood for each study subject. We chose blood as the biologic indicator of arsenic exposure to estimate better the relation between arsenic challenge and the resultant oxidative stress for organs and tissues in the body. Once digested as a water solution, arsenic is rapidly transported by the blood to such organs as the liver, kidneys, lungs, intestines, and the skin within 24 hr (34). Although 90% of the bloodborne arsenic is rapidly cleared (34), blood arsenic level is still a useful indicator of continuous arsenic exposure (35). In contrast, urine arsenic is the best indicator of recent exposure of several days, and hair or even fingernail concentrations of arsenic can reflect recent exposure of several months (36). However, these latter two biologic media for determining arsenic exposure suffer a time-lag effect for assessment of their relation to labile radicals formed in study subjects.

To determine the arsenic concentration in whole blood of study subjects, we used a flame atomic absorption spectrophotometric method developed by Wang et al. (37) with slight modification. Briefly, 2 mL of concentrated  $\text{HNO}_3$  was added to 1 mL of whole blood in a digestion flask. The digestion was maintained at  $100^{\circ}\text{C}$  for 40 min. After addition of 0.7 mL each of concentrated  $\text{H}_2\text{SO}_4$  and  $\text{HClO}_4$ , digestion was continued for another 60 min. Finally, 1 mL of high-purity water was added and heated until a colorless solution was obtained. The solution was then diluted to 12 mL and quantified

for total arsenic using an atomic absorption spectrophotometer model Z-8000 and its accessory Hydride Formation System HFS-2 (Hitachi, Tokyo, Japan). According to a recovery test for arsenic determination in this study, the recovery of arsenic added to ion-free water was 83.2%, and the interassay variation was 7% (replications = 6).

**Measurement of the level of reactive oxidants in plasma.** We wrapped heparinized blood samples obtained from study subjects in aluminum foil to prevent light exposure until testing for reactive oxidant levels. To measure the production of oxygen free radicals in samples, we adopted a chemiluminescence method, with slight modification, using lucigenin (1 mg/mL) as an amplifier for measuring superoxide ( $\text{O}_2^-$ ) (38–40). Briefly, the blood sample was first centrifuged to separate plasma from packed cells, and 80 µL of the plasma was immediately placed in a 96-well dish for oxidative stress assay using a chemiluminescence analyzer (TopCount System; Packard, Meriden, CT, USA). After adding lucigenin, we counted photon emission from the sample at 10-sec intervals at room temperature under atmospheric conditions. In a preliminary experiment, the chemiluminescence level responded immediately after addition of 200 µL lucigenin [(Sigma, St. Louis, MO, USA) 1 mg/mL in phosphate-buffered saline] and approached its maximum at 5 min. Afterward, a steady level lasted for 10 min without a significant change. Thus, the chemiluminescence measurement for each sample was determined at 5 min after addition of lucigenin to the plasma. For each sample, the assay was performed in triplicate, and the reactive oxidant level was expressed as mean chemiluminescence intensity counts (counts per 5 min). All samples were processed in the dark.

**Measurement of antioxidant capacity level in plasma.** Plasma was separated within 6 hr of collection by centrifugation and preserved at  $-20^{\circ}\text{C}$  for antioxidant capacity assay. We measured plasma antioxidant capacity within 1 week using the 2,2'-azino-di[3-ethylbenzthiazoline] sulfonate (ABTS) assay method (Radox Laboratories, Antrim, UK). In this assay, incubation of ABTS with

**Table 1.** Distribution of age, sex, and arsenic content in blood by arsenic concentration in well water among 64 residents of the Lanyang Basin, northeastern Taiwan, 1997–1998.

Characteristic	Total number	Arsenic concentration in well water (µg/L)			
		$\leq 10$ (n = 15)	10.1–50 (n = 13)	50.1–300 (n = 15)	$> 300$ (n = 21)
Mean age in years (range)	64	56.7 (42–75)	58.6 (45–67)	60.0 (42–71)	54.8 (42–67)
Sex (%)					
Male	26	6 (40)	5 (62)	6 (40)	9 (43)
Female	38	9 (60)	8 (38)	9 (60)	12 (57)
Mean arsenic content in blood, µg/L (SD)	64	7.2 (8.4)	8.1 (12.9)	10.8 (10.0)	11.4 (9.1)

H<sub>2</sub>O<sub>2</sub> and a peroxidase (metmyoglobin) produced the blue-green radical cation ABTS<sup>+</sup>, which was measured at a wavelength of 600 nm. Antioxidants in test plasma dose-dependently suppressed this color production. The system was standardized using Trolox, a water-soluble vitamin E analogue. The assay for each sample was performed in triplicate, and results are expressed as mean millimoles per liter.

**Genetic indicators of arsenic metabolism capability.** We considered two genetic categories encoding enzymes implicated in arsenic metabolism for this study: One involves methyltransferases of arsenic species; the other is the glutathione *S*-transferase (GST) supergene family. Because sequence information of arsenic methyltransferases is not yet available in the literature, it is impossible to investigate the polymorphisms of these enzymes (41). Instead, arsenic species and their metabolites—including arsenite, arsenate, monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA)—excreted in urine are often studied to reflect personal arsenic methylation capability (42). In the present study, we analyzed urinary speciation for each study subject, and the results were published in a previous report (43). To indicate an individual's primary and secondary methylation capability in arsenic metabolism, we used ratios of MMA to arsenite combined with arsenate (MMA/InAs) and of DMA to MMA (DMA/MMA), respectively.

GSTP1 enzyme has been implicated in arsenic metabolism (44), and the genetic polymorphism at the *GSTP1* locus is suggested as an important factor in cancer etiology (45). To determine the genotype at exon 5 of the *GSTP1* gene for study subjects, DNA was extracted from samples of peripheral blood cells collected in 1995 (33), and assayed for this study using the polymerase chain reaction–restriction fragment length

polymorphism method (46). We first identified two variant *GSTP1* cDNAs representing alleles of 105 isoleucine (Ile) and 105 valine (Val) forms of the *GSTP1* protein. We then classified study subjects into Ile/Ile, Ile/Val, or Val/Val genotypes. *GSTP1* activities are lowest among Val/Val homozygotes, intermediate among Ile/Val heterozygotes, and highest among Ile/Ile homozygotes (46).

**Statistical analyses.** Each individual oxygen free radical measurement was logarithm-transformed to stabilize the variance and to cause the distribution to approach normality. All oxygen free radical values presented in the figures and tables are logarithm-transformed. We used the analysis of variance (ANOVA) F-test to examine the statistical significance of differences in the assayed end points between groups of risk factors. We determined associations between study variables by Pearson's product-moment correlation coefficients (*r*). We performed linear regression analysis to examine the effect of arsenic concentration in whole blood on plasma oxidative stress level and total antioxidant capacity after controlling for confounding factors. The level of statistical significance was *p* < 0.05.

## Results

### Characteristics of the study population.

Table 1 shows the age and sex distribution of study subjects by level of arsenic concentration in well water. We recruited 64 study subjects (80% response rate) for this study. The age of participants ranged from 42 to 75 years, with 26 males and 38 females. The mean and SD of arsenic content in whole blood of each exposure group are also presented in Table 1. People who had used well water with higher arsenic levels had a higher level of arsenic in their whole blood.

The frequency distribution of (logarithm-transformed) plasma reactive oxidant levels and antioxidant capacity levels for

study participants are graphed in Figure 1. The plasma reactive oxidants and antioxidant capacity stratified by age, sex, educational level, and main occupation are presented in Table 2. In general, the mean levels of plasma reactive oxidants and antioxidant capacity did not significantly differ among groups stratified by sex, educational level, or main occupation. In contrast, plasma antioxidant capacity decreased with increased age ( $\beta$ -coefficient =  $-0.0045$ , *p* = 0.014).

### Relationship of reactive oxidants to blood arsenic levels.

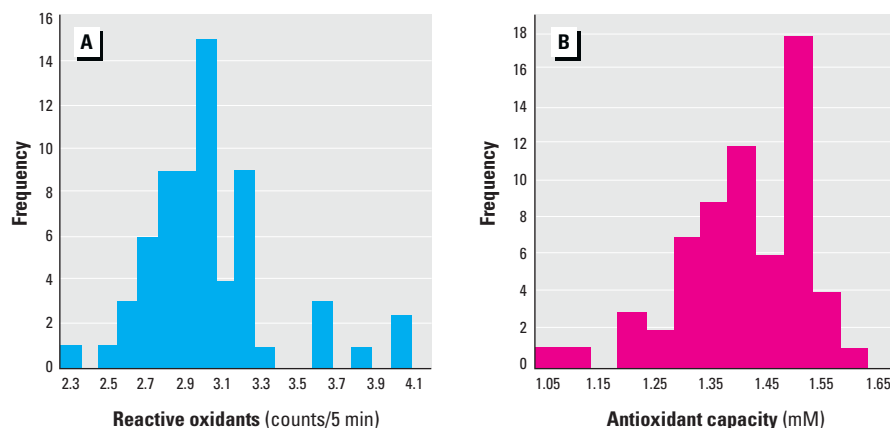
The crude association between levels of plasma reactive oxidants and arsenic concentration in whole blood is illustrated in Figure 2A. The reactive oxidant level was significantly correlated with arsenic concentration in whole blood of study subjects ( $r = +0.41$ , *p* = 0.001). The reactive oxidant level increased 0.013 counts/5 min (antilog = 1.03 counts) per 1  $\mu\text{g/L}$  increase in blood arsenic level (*p* = 0.001). Other study factors, including cigarette smoking and alcohol and tea consumption, as well as three indicators of arsenic metabolism capability—the ratio of MMA to InAs, ratio of DMA to MMA, and *GSTP1* genotype—were not significantly (*p* > 0.05) correlated to the level of plasma reactive oxidants (Table 3).

Previous studies have suggested that oxidative lesions in DNA accumulate with age and are inversely associated with individual antioxidant capacity (29). We thus further adjusted for the aging effect and diminished antioxidant capacity in our regression analyses. As shown in Models I and II of Table 4, the association of reactive oxidant level with arsenic content in whole blood did not change substantially when age and antioxidant capacity were included in the analysis.

### Relationship of antioxidant capacity to blood arsenic levels.

The distribution of antioxidant capacity levels in relation to arsenic concentration in whole blood is graphed in Figure 2B. Despite the wide variation in individual antioxidant capacity, we observed a significant inverse correlation of plasma antioxidant capacity with arsenic concentration in whole blood ( $r = -0.30$ , *p* = 0.014). The plasma antioxidant capacity level decreased 0.0034 mM per 1  $\mu\text{g/L}$  increase in arsenic content in whole blood (*p* = 0.014) as shown in the  $\beta$ -coefficient value of the regression line.

As shown in Table 3, there were no significant (*p* > 0.05) associations of plasma antioxidant capacity with lifestyle characteristics, including cigarette smoking and alcohol and tea consumption. Neither the ratio of DMA to MMA nor the *GSTP1* genotype was associated with plasma antioxidant



**Figure 1.** Frequency distributions of levels of plasma (A) reactive oxidants and (B) antioxidant capacity among 64 residents consuming arsenic-contaminated well water in the Lanyang Basin, northeastern Taiwan. The (logarithm-transformed) reactive oxidant level was detected by a chemiluminescence assay measuring the superoxide anion. Antioxidant capacity level was measured using the ABTS method.

capacity in study subjects ( $p > 0.05$ ). On the other hand, we observed a significant positive association between plasma antioxidant capacity and the ratio of MMA to InAs in urine ( $p = 0.029$ ). In other words, the lower the primary arsenic methylation capability, the lower the antioxidant capacity level in plasma. However, the decreased level of plasma antioxidant capacity caused by blood arsenic remained significant after adjustment for age and primary arsenic methylation capability (Models III and IV of Table 4).

## Discussion

Our data show that arsenic concentration in whole blood of individuals is positively associated with the level of reactive oxidants and negatively associated with the antioxidant capacity level in plasma. The present results, consistent with what we observed in *in vitro* studies, provide evidence that drinking arsenic-contaminated well water may increase the levels of oxidative stress in peripheral blood in humans. The increased level of reactive oxygen radicals in plasma may represent the net result of increased radical production and decreased antioxidant activity. However, in this study, the positive association between arsenic content in blood and oxygen free radicals in plasma was only partially attributable to the inverse correlation of blood arsenic to plasma antioxidants. These results suggest that arsenic digestion in study subjects enhances the formation of oxygen free radicals in plasma and reduces the antioxidant capacity of arsenic-ingesting subjects.

The free radical theory of arsenic toxicity has recently been gaining acceptance in cell culture studies. Further, the superoxide anion and hydroxy peroxide ( $H_2O_2$ ) are the predominant reactive species produced by cultured cells in response to exposure to arsenite (16,47,48). Our study demonstrates that changes in lucigenin-derived chemiluminescence levels measuring superoxide in plasma were positively associated with arsenic concentration in whole blood of study subjects. These data are consistent with the results of previous studies. However, the detection of superoxide present in plasma of study subjects by the chemiluminescence method may very likely underestimate the levels of reactive oxygen species caused by arsenic ingestion. Nevertheless, the presence of superoxide in plasma is consequential, as shown by its significant association with blood arsenic concentrations in study subjects.

The ABTS method we used in this study to quantitate antioxidant capacity uses the ability of test plasma to inhibit the generation of free radicals by metmyoglobin and hydrogen peroxide. Previous studies with cultured cells have shown that several antioxidant activities, such as those of superoxide

dismutase (SOD) and catalase, may be modulated by sodium arsenite, thus accumulating superoxide and  $H_2O_2$ , respectively (16,27). In addition to these cellular antioxidant enzymes, nonenzymatic antioxidants, such as glutathione (GSH), bilirubin, ferritin, and uric acid, as well as exogenous antioxidant molecules such as  $\alpha$ -tocopherol,  $\beta$ -carotene, and ascorbic acid, also provide primary defense against extracellular and intracellular free radicals (49). In this study, we observed that subjects with higher arsenic content in whole blood had lower antioxidant capacity in plasma. The antioxidants measured in plasma of subjects in this study should represent the components in the extracellular environment, where levels of SOD, catalase, GSH, and GSH peroxidase are often very low (50). Thus, transport or storage proteins, which inactivate the radical generation activities of transition metals by

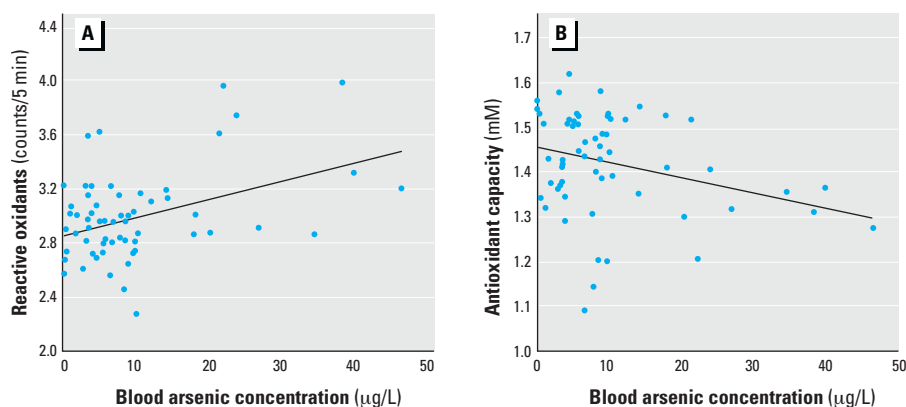
sequestering them, as well as supplementary vitamins may have been the targets for the assay in this study. The individual components of these extracellular antioxidants and the ways the antioxidants are suppressed by ingested arsenic need further elucidation in future studies.

Results of the present study also show that the level of antioxidant capacity in plasma declined with the age of study subjects. The effect of aging on antioxidant activity has been controversial, depending upon the populations studied and antioxidants measured (51–54). In this study, plasma antioxidant capacity was measured by the ABTS method, which assays the total capacity of antioxidant defense. Because antioxidant defense exists as a balanced and coordinated system, the total capacity of the antioxidant defense may give a more precise indication of the relationship between the

**Table 2.** Plasma reactive oxidant and antioxidant capacity levels by demographic characteristics among 64 residents of the Lanyang Basin, northeastern Taiwan, 1997–1998.

Characteristic	Number	Reactive oxidants level <sup>a</sup>		Antioxidant capacity level <sup>b</sup>	
		Mean $\pm$ SD	<i>p</i> -Value <sup>c</sup>	Mean $\pm$ SD	<i>p</i> -Value
Total subjects	64	3.001 $\pm$ 0.320		1.424 $\pm$ 0.112	
Age groups (years)					
< 50	13	3.003 $\pm$ 0.384		1.473 $\pm$ 0.095	
50–59	25	3.091 $\pm$ 0.341		1.447 $\pm$ 0.092	
$\geq 60$	26	2.913 $\pm$ 0.244	0.141	1.377 $\pm$ 0.124	0.016
Sex					
Male	26	2.965 $\pm$ 0.315		1.428 $\pm$ 0.107	
Female	38	3.025 $\pm$ 0.325	0.467	1.417 $\pm$ 0.121	0.702
Education level					
Illiterate	17	3.001 $\pm$ 0.300		1.418 $\pm$ 0.116	
Elementary	45	2.990 $\pm$ 0.324		1.428 $\pm$ 0.114	
Junior high and above	2	3.231 $\pm$ 0.520	0.589	1.383 $\pm$ 0.040	0.831
Main occupation					
Retired	22	2.998 $\pm$ 0.316		1.418 $\pm$ 0.122	
Farming	28	2.929 $\pm$ 0.282		1.421 $\pm$ 0.122	
Others	14	3.149 $\pm$ 0.368	0.109	1.438 $\pm$ 0.077	0.862

<sup>a</sup>Reactive oxidant level (logarithm-transformed) was detected by a chemiluminescence assay measuring the superoxide anion (counts/5 min). <sup>b</sup>Antioxidant capacity level was measured using the ABTS method (mM). <sup>c</sup>Probability derived from an ANOVA F-test for the hypothesis that there is no difference between groups.



**Figure 2.** Correlations of levels of plasma (A) reactive oxidants ( $y = 0.013x + 2.8755$ ) and (B) antioxidant capacity ( $y = -0.0034x + 1.4568$ ) with arsenic concentrations in whole blood among 64 residents of the Lanyang Basin, northeastern Taiwan, 1997–1998. The (logarithm-transformed) reactive oxidant level was detected by a chemiluminescence assay measuring the superoxide anion;  $r = 0.41$ ,  $p = 0.001$ . Antioxidant capacity level was measured using the ABTS method;  $r = -0.30$ ,  $p = 0.014$ .

assayed end point and the risk factors. The total antioxidant capacity of the study subjects in this study decreased 0.0044 mM per yearly increment in age after adjusting for blood arsenic (Table 4). Interestingly, the total antioxidant capacity among Chinese in the geographic area of the present Taiwan study is generally lower than that of a Hong Kong Chinese population assayed in a previous study by other investigators (51). The combined effect of aging and chronic exposure to arsenic of the participants in our study may explain the difference.

We found no significant correlation of lifestyle characteristics, such as cigarette smoking and alcohol and tea consumption, with reactive oxidants level or antioxidant capacity

level in plasma. Smoking-related increases in oxidized products including nitric oxides and lipid peroxides have been reported previously (55,56). The nature of the lucigenin-based chemiluminescence assay system for superoxide anion or some unidentified characteristics of study subjects in this study may explain the discrepancy of finding no smoking-related oxidative stress. Ethanol has also been reported to affect oxidative stress level during its metabolism (57,58). Tea components have anticancer properties and act as free-radical scavengers (59,60). Only a small number of the subjects consumed alcohol or tea in the present study, so random variation due to the small sample size may have occurred.

Among the indicators of arsenic metabolism capability, primary arsenic methylation capability indexed by the ratio of MMA/InAs had a positive association with plasma antioxidant capacity even when adjusted further for the aging effect and arsenic concentration in whole blood. This result indicates that a high capacity for methylating InAs into MMA may alleviate depletion of the antioxidant system; that is, MMA lowers the antioxidant capacity level in plasma less than does InAs. This is consistent with the notion that organic arsenic is usually less toxic than InAs compounds. To elucidate the molecular mechanism of this methyltransformation, the genetic polymorphisms of arsenite methyltransferase must be identified.

GST consists of a large family of xenobiotic-metabolizing enzymes that catalyze the conjugation of reduced GSH to a vast array of hydrophobic and electrophilic compounds, as well as remove compounds that can generate reactive oxygen species (61). GSH has been suggested to be a necessary component for arsenic metabolism probably in the initial reduction of arsenate to arsenite and in subsequent oxidative methylation (62,63). In the present study, the results show that individuals with the 105 Val allele (i.e., lower GSTP1-conjugating activity) had a higher level of oxidative stress and a lower level of antioxidant capacity in plasma (shown in Table 3). However, differences were not statistically significant. The *GSTP1* genotype alone may confer a relatively weak influence, which may be masked by unidentified confounding factors.

Excessive oxidative stress has long been proposed to cause deleterious effects on biologic systems; however, it has been difficult to prove its association with the development of cancers and cardiovascular diseases in humans. High levels of oxidized products or low levels of antioxidant status have been found sporadically in patients with relevant disease (29–31, 64). Oxidized products detected in target tissues often represent a small spectrum of the total DNA damage, including results from reactive radicals. Furthermore, a lack of specificity often limits the use of oxidized products in population studies. In the present study, we measured varying levels of arsenic content in blood in healthy study subjects with no known inflammation. Oxidative stress, indicated by blood superoxide level, had a good association with the level of blood arsenic among study subjects. Because blood flows to all organs, an elevated level of oxidative stress in whole blood may exert a pathogenic effect in target tissues. Arsenic-induced oxidants, such as the superoxide anion and hydroxy peroxide, have been implicated in *in vitro* studies (16,27). These oxidants are suggested to

**Table 3.** Plasma reactive oxidant and antioxidant capacity levels by lifestyle characteristics and arsenic metabolism capability among 64 residents of the Lanyang Basin, northeastern Taiwan, 1997–1998.

Characteristic	Number <sup>a</sup>	Reactive oxidant level <sup>b</sup>		Antioxidant capacity level <sup>c</sup>	
		Mean ± SD	p-Value <sup>d</sup>	Mean ± SD	p-Value
Lifestyle characteristic					
Cigarette smoking					
No	44	2.995 ± 0.345	0.834	1.432 ± 0.106	0.372
Yes	20	3.013 ± 0.318		1.405 ± 0.127	
Alcohol consumption					
No	59	2.991 ± 0.320	0.439	1.428 ± 0.108	0.321
Yes	5	3.108 ± 0.329		1.376 ± 0.166	
Tea consumption					
No	58	2.994 ± 0.325	0.618	1.421 ± 0.116	0.484
Yes	6	3.063 ± 0.284		1.455 ± 0.065	
Arsenic metabolism capability					
MMA/InAs ratio					
< 1.8	20	3.072 ± 0.424	0.209	1.380 ± 0.112	0.029
1.8–3.0	21	2.907 ± 0.190		1.449 ± 0.113	
≥ 3.0	21	3.047 ± 0.306		1.462 ± 0.077	
DMA/MMA ratio					
< 1.9	21	3.034 ± 0.337	0.710	1.468 ± 0.083	0.156
1.9–2.9	20	3.030 ± 0.191		1.412 ± 0.109	
≥ 2.9	21	2.960 ± 0.408		1.412 ± 0.120	
<i>GSTP1</i> genotype					
Ile/Ile	40	2.962 ± 0.240	0.124	1.426 ± 0.112	0.556
Ile/Val or Val/Val	20	3.101 ± 0.450		1.407 ± 0.121	

<sup>a</sup>There were two subjects without data on arsenic metabolism capability, and four subjects without data on *GSTP1* genotype. <sup>b</sup>Reactive oxidant level (logarithm-transformed) was detected by a chemiluminescence assay measuring the superoxide anion (counts/5 min). <sup>c</sup>Antioxidant capacity level was measured using the ABTS method (mM). <sup>d</sup>Probability derived from an ANOVA F-test for the hypothesis that there is no difference between groups.

**Table 4.** Multiple linear regression analyses of plasma reactive oxidant and antioxidant capacity levels among 64 residents of the Lanyang Basin, northeastern Taiwan, 1997–1998.

Model	Variable	Coefficient (× 100)	SE <sup>a</sup> (× 100)	p-Value <sup>b</sup>
Reactive oxidants level <sup>c</sup>				
I	Age (1-year increment)	−0.61	0.48	0.214
	Arsenic content in blood (1-μg/L increment)	1.31	0.37	0.001
	Age (1-year increment)	−0.79	0.51	0.127
	Total antioxidant capacity (1-mM increment)	−40.77	36.33	0.266
II	Arsenic content in blood (1-μg/L increment)	1.17	0.39	0.004
Antioxidant capacity level <sup>d</sup>				
III	Age (1-year increment)	−0.44	0.17	0.012
	Arsenic content in blood (1-μg/L increment)	−0.34	0.13	0.012
IV	Age (1-year increment)	−0.35	0.16	0.037
	MMA/InAs (< 1.8 vs. 1.8–3.0)	6.21	2.97	0.041
	MMA/InAs (< 1.8 vs. ≥ 3.0)	7.86	2.95	0.010
	Arsenic content in blood (1-μg/L increment)	−0.32	0.12	0.010

<sup>a</sup>SE of the coefficient, SD/(N−1)<sup>0.5</sup>; n = 64. <sup>b</sup>Probability derived from a Wald's chi-square test for the hypothesis that the regression coefficient = 0. <sup>c</sup>Reactive oxidant level (logarithm-transformed) was detected by a chemiluminescence assay measuring the superoxide anion (counts/5 min). <sup>d</sup>Antioxidant capacity level was measured using the ABTS method (mM).

damage macromolecules in cells or to act as second messengers, leading to alteration of the gene expression profile in cells and subsequent enhancement of cell proliferation (65,66). The positive association of reactive oxygen radicals with arsenic content in blood found in this study may explain why arsenic induces both cancers and atherosclerotic lesions at several anatomic sites, as we observed previously among residents of the arseniasis-endemic area (5,67). Arsenic also reduces antioxidant levels in plasma, which may accelerate disease development at target sites. This contention is consistent with observations of previous studies that levels of  $\beta$ -carotene were lower in patients with arsenic-induced skin cancer (68) as well as in patients with ischemic heart disease (69) than in healthy controls.

In summary, we present evidence that arsenic in blood is not only associated with an increased level of reactive oxygen radicals but is also inversely related to the antioxidant capacity in plasma of humans. The results of this study indicate that arsenic is a significant environmental toxicant that increases the risk of oxidative stress in exposed persons. Persistent high levels of oxidative stress may be a mechanism underlying the carcinogenesis and atherosclerosis induced by long-term arsenic exposure.

## REFERENCES AND NOTES

- Thornton I, Farago M. The geochemistry of arsenic. In: *Arsenic: Exposure and Health Effects* (Abernathy CO, Calderon RL, Chappell WR, eds). London:Chapman & Hall, 1997;1-16.
- WHO. Environmental Health Criteria 18: Arsenic. Geneva: World Health Organization, 1981.
- U.S. PHS. Toxicological Profile for Arsenic. Washington DC:U.S. Public Health Service, 1989.
- Bates MN, Smith AH, Hopenhayn Rich C. Arsenic ingestion and internal cancers: a review. *Am J Epidemiol* 135:462-476 (1992).
- Chiou HY, Hsueh YM, Liaw KF, Horng SF, Chiang MH, Pu YS, Lin JS, Huang CH, Chen CJ. Incidence of internal cancers and ingested inorganic arsenic: a seven-year follow-up study in Taiwan. *Cancer Res* 55:1296-1300 (1995).
- Wu HY, Chen KP, Tseng WP, Hsu CL. Epidemiologic studies on blackfoot disease: I. prevalence and incidence of the disease by age, sex, year, occupation and geographical distribution. *Mem Coll Med Natl Taiwan Univ* 7:33-50 (1961).
- Chen CJ, Wu MM, Lee SS, Wang JD, Cheng SH, Wu HY. Atherogenicity and carcinogenicity of high-arsenic artesian well water. Multiple risk factors and related malignant neoplasms of blackfoot disease. *Arteriosclerosis* 8:452-460 (1988).
- Wu MM, Kuo TL, Hwang YH, Chen CJ. Dose-response relation between arsenic concentration in well water and mortality from cancers and vascular diseases. *Am J Epidemiol* 130:1123-1132 (1989).
- Chiou HY, Huang WI, Su CL, Chang SF, Hsu YH, Chen CJ. Dose-response relationship between prevalence of cerebrovascular disease and ingested inorganic arsenic. *Stroke* 28:1717-1723 (1997).
- Lilienfeld DE. Arsenic, geographical isolates, environmental epidemiology, and arteriosclerosis [Editorial]. *Arteriosclerosis* 8:449-451 (1988).
- Lee TC, Oshimura M, Barrett JC. Comparison of arsenic-induced cell transformation, cytotoxicity, mutation and cytogenetic effects in Syrian hamster embryo cells in culture. *Carcinogenesis* 6:1421-1426 (1985).
- Huang RN, Ho IC, Yih LH, Lee TC. Sodium arsenite induces chromosome endoreduplication and inhibits protein phosphatase activity in human fibroblasts. *Environ Mol Mutagen* 25:188-196 (1995).
- Yih LH, Ho IC, Lee TC. Sodium arsenite disturbs mitosis and induces chromosome loss in human fibroblasts. *Cancer Res* 57:5051-5059 (1997).
- Jacobson-Kram D, Montalbano D. The reproductive effects assessment group's report on the mutagenicity of inorganic arsenic. *Environ Mutagen* 7:787-804 (1985).
- Lee TC, Tanaka N, Lamb PW, Gilmer TM, Barrett JC. Induction of gene amplification by arsenic. *Science* 241:79-81 (1988).
- Wang TS, Huang H. Active oxygen species are involved in the induction of micronuclei by arsenite in XRS-5 cells. *Mutagenesis* 9:253-257 (1994).
- Yih LH, Lee TC. Effects of exposure protocols on induction of kinetochore-plus and -minus micronuclei by arsenite in diploid human fibroblasts. *Mutat Res* 440:75-82 (1999).
- Lee TC, Huang RY, Jan KY. Sodium arsenite enhances the cytotoxicity, clastogenicity, and 6-thioguanine-resistant mutagenicity of ultraviolet light in Chinese hamster ovary cells. *Mutat Res* 148:83-89 (1985).
- Lee TC, Lee KC, Tzeng YJ, Huang RY, Jan KY. Sodium arsenite potentiates the clastogenicity and mutagenicity of DNA crosslinking agents. *Environ Mutagen* 8:119-128 (1986).
- Lee TC, Wang-Wuu S, Huang RY, Lee KC, Jan KY. Differential effects of pre- and posttreatment of sodium arsenite on the genotoxicity of methyl methanesulfonate in Chinese hamster ovary cells. *Cancer Res* 46:1854-1857 (1986).
- Barchowsky A, Dudek EJ, Treadwell MD, Wetterhahn KE. Arsenic induces oxidant stress and NF-kappa B activation in cultured aortic endothelial cells. *Free Radic Biol Med* 21:783-790 (1996).
- Cavigelli M, Li WW, Lin A, Su B, Yoshioka K, Karin M. The tumor promoter arsenite stimulates AP-1 activity by inhibiting a JNK phosphatase. *EMBO J* 15:6269-6279 (1996).
- Liu Y, Guyton KZ, Gorospe M, Xu Q, Lee JC, Holbrook NJ. Differential activation of ERK, JNK/SAPK and P38/CBP/RK map kinase family members during the cellular response to arsenite. *Free Radic Biol Med* 21:771-781 (1996).
- Pinkus R, Weiner LM, Daniel V. Role of oxidants and antioxidants in the induction of AP-1, NF-kappaB, and glutathione S-transferase gene expression. *J Biol Chem* 271:13422-13429 (1996).
- Nordenson I, Beckman L. Is the genotoxic effect of arsenic mediated by oxygen free radicals? *Hum Hered* 41:71-73 (1991).
- Yamanaka K, Hasegawa A, Sawamura R, Okada S. Cellular response to oxidative damage in lung induced by the administration of dimethylarsinic acid, a major metabolite of inorganic arsenics, in mice. *Toxicol Appl Pharmacol* 108:205-213 (1991).
- Lee TC, Ho IC. Modulation of cellular antioxidant defense activities by sodium arsenite in human fibroblasts. *Arch Toxicol* 69:498-504 (1995).
- Lynn S, Shiung JN, Gurr JR, Jan KY. Arsenite stimulates poly(ADP-ribosylation) by generation of nitric oxide. *Free Radic Biol Med* 24:442-449 (1998).
- Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci USA* 90:7915-7922 (1993).
- Feig DI, Reid TM, Loeb LA. Reactive oxygen species in tumorigenesis. *Cancer Res* 54:1890s-1894s (1994).
- Wiseman H, Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem J* 313:17-29 (1996).
- Chen CJ, Wang CJ. Ecological correlation between arsenic level in well water and age-adjusted mortality from malignant neoplasms. *Cancer Res* 50:5470-5474 (1990).
- Chiou HY. Epidemiological Studies on Inorganic Arsenic Methylation Capacity and Inorganic Arsenic Induced Health Effects Among Residents in the Blackfoot Disease Endemic Area and Lanyang Basin in Taiwan [PhD Thesis]. Taipei, Taiwan:National Taiwan University, 1996.
- Vahter M. Metabolism of arsenic. In: *Biological and Environmental Effects of Arsenic* (Fowler BA, ed). Amsterdam:Elsevier Science Publishing Co., 1983;171-198.
- Morton WE, Dunnette DA. Health effects of environmental arsenic. In: *Arsenic in the Environment. Part II: Human Health and Ecosystem Effects* (Nriagu JO, ed). New York:John Wiley & Sons, Inc., 1994;17-34.
- Goyer RA. Toxic effects of metals. In: *Casarett and Doull's Toxicology: The Basic Science of Poisons* (Klaassen CD, Amdur MO, Doull J, eds). New York: MacMillan Publishing Co., 1986;582-635.
- Wang CT, Huang CW, Chou SS, Lin DT, Liao SR, Wang RT. Studies on the concentration of arsenic, selenium, copper, zinc and iron in the blood of blackfoot disease patients in different clinical stages. *Eur J Clin Chem Clin Biochem* 31:759-763 (1993).
- Lu FJ, Lin JT, Wang HP, Huang WC. A simple, sensitive, non-stimulated photon counting system for detection of superoxide anion in whole blood. *Experientia* 52:141-144 (1996).
- Sun JS, Hang YS, Huang IH, Lu FJ. A simple chemiluminescence assay for detecting oxidative stress in ischemic limb injury. *Free Radic Biol Med* 20:107-112 (1996).
- Vladimirov YA. Intrinsic (low-level) chemiluminescence. In: *Free Radicals. A Practical Approach* (Punchard NA, Kelly FJ, eds). New York:Oxford University Press, 1996;65-82.
- Aposhian HV. Enzymatic methylation of arsenic species and other new approaches to arsenic toxicity. *Annu Rev Pharmacol Toxicol* 37:397-419 (1997).
- U.S. EPA. Special Report on Ingested Inorganic Arsenic. Skin Cancer. Nutritional Essentiality. EPA 625/3-87/013. Washington, DC:U.S. Environmental Protection Agency, 1988.
- Chiou HY, Hsueh YM, Hsieh LL, Hsu LI, Hsu YH, Hsieh FI, Wei ML, Chen HC, Yang HT, Leu LC, et al. Arsenic methylation capacity, body retention, and null genotypes of glutathione S-transferase M1 and T1 among current arsenic-exposed residents in Taiwan. *Mutat Res* 386:197-207 (1997).
- Lo JF, Wang HF, Tam MF, Lee TC. Glutathione S-transferase pi in an arsenic-resistant Chinese hamster ovary cell line. *Biochem J* 288:977-982 (1992).
- Hengstler JG, Arand M, Herrero ME, Oesch F. Polymorphisms of N-acetyltransferases, glutathione S-transferases, microsomal epoxide hydrolase and sulfotransferases: influence on cancer susceptibility. *Recent Results Cancer Res* 154:47-85 (1998).
- Watson MA, Stewart RK, Smith GB, Massey TE, Bell DA. Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. *Carcinogenesis* 19:275-280 (1998).
- Chen MF, Mo LR, Lin RC, Kuo JY, Chang KK, Liao C, Lu FJ. Increase of resting levels of superoxide anion in the whole blood of patients with decompensated liver cirrhosis. *Free Radic Biol Med* 23:672-679 (1997).
- Barchowsky A, Klei LR, Dudek EJ, Swartz HM, James PE. Stimulation of reactive oxygen, but not reactive nitrogen species, in vascular endothelial cells exposed to low levels of arsenite. *Free Radic Biol Med* 27:1405-1412 (1999).
- Dreher D, Junod AF. Role of oxygen free radicals in cancer development. *Eur J Cancer* 32A:30-38 (1996).
- Halliwell B, Gutteridge JM. The antioxidants of human extracellular fluids. *Arch Biochem Biophys* 280:1-8 (1994).
- Woo J, Leung SS, Lam CW, Ho SC, Lam TH, Janus ED. Plasma total antioxidant capacity in an adult Hong Kong Chinese population. *Clin Biochem* 30:553-557 (1997).
- King CM, Bristow Craig HE, Gillespie ES, Barnett YA. *In vivo* antioxidant status, DNA damage, mutation and DNA repair capacity in cultured lymphocytes from healthy 75- to 80-year-old humans. *Mutat Res* 377:137-147 (1997).
- Benzi G, Moretti A. Age- and peroxidative stress-related modifications of the cerebral enzymatic activities linked to mitochondria and the glutathione system. *Free Radic Biol Med* 19:77-101 (1995).
- Harris ED. Regulation of antioxidant enzymes. *FASEB J* 6:2675-2683 (1992).
- Cosgrove JP, Borish ET, Church DF, Pryor WA. The metal-mediated formation of hydroxyl radical by aqueous extracts of cigarette tar. *Biochem Biophys Res Commun* 132:390-396 (1985).
- Frei B, Forte TM, Ames BN, Cross CE. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. Protective effects of ascorbic acid. *Biochem J* 277:133-138 (1991).
- Mufti SI. Alcohol acts to promote incidence of tumors. *Cancer Detect Prev* 16:157-162 (1992).

58. Ishii H, Kurose I, Kato S. Pathogenesis of alcoholic liver disease with particular emphasis on oxidative stress. *J Gastroenterol Hepatol* 12:S272–282 (1997).
59. Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Rice-Evans C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch Biochem Biophys* 322:339–346 (1995).
60. Dong Z, Ma W, Huang C, Yang CS. Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (-)-epigallocatechin gallate, and theaflavins. *Cancer Res* 57:4414–4419 (1997).
61. Sipes IG, Gandolfi AJ. Biotransformation of toxicants. In: Casarett and Doull's Toxicology: The Basic Science of Poisons (Klaassen CD, Amdur MO, Doull J, eds). New York: Macmillan Publishing Co., 1986;64–98.
62. Georis B, Cardenas A, Buchet JP, Lauwerys R. Inorganic arsenic methylation by rat tissue slices. *Toxicology* 63:73–84 (1990).
63. Thompson DJ. A chemical hypothesis for arsenic methylation in mammals. *Chem Biol Interact* 88:89–114 (1993).
64. Tse WY, Maxwell SR, Thomason H, Blann A, Thorpe GH, Waite M, Holder R. Antioxidant status in controlled and uncontrolled hypertension and its relationship to endothelial damage. *J Hum Hypertens* 8:843–849 (1994).
65. Farber JL. Mechanisms of cell injury by activated oxygen species. *Environ Health Perspect* 102(suppl 10):17–24 (1994).
66. Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radic Biol Med* 18:775–794 (1995).
67. Chen CJ, Chuang YC, Lin TM, Wu HY. Malignant neoplasms among residents of a blackfoot disease-endemic area in Taiwan: high-arsenic artesian well water and cancers. *Cancer Res* 45:5895–5899 (1985).
68. Hsueh YM, Chiou HY, Huang YL, Wu WL, Huang CC, Yang MH, Lue LC, Chen GS, Chen CJ. Serum beta-carotene level, arsenic methylation capability, and incidence of skin cancer. *Cancer Epidemiol Biomarkers Prev* 6:589–596 (1997).
69. Hsueh YM, Wu WL, Huang YL, Chiou HY, Tseng CH, Chen CJ. Low serum carotene level and increased risk of ischemic heart disease related to long-term arsenic exposure. *Atherosclerosis* 141:249–257 (1998).